

Efficient Transfection of the Archaeobacterium *Halobacterium halobium*

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We developed an efficient polyethylene glycol-mediated spheroplast transfection method for the extremely halophilic archaeobacterium *Halobacterium halobium*. The 59-kilobase-pair linear phage Φ H DNA molecule routinely produced between 5×10^6 and 2×10^7 transfectants per μ g of DNA. Between 0.5 and 1% of spheroplasts were transfected per μ g of Φ H DNA. Under our conditions, survival and regeneration of *H. halobium* spheroplasts were also quite efficient, suggesting that this method will be useful for introducing other DNAs into these bacteria.

It has been almost a decade since Woese and Fox (13, 15) first observed that certain procaryotes (the methanogens, extreme halophiles, and sulfur-dependent thermoacidophiles) share common molecular traits of extraordinarily ancient derivation which qualitatively differ from the characteristics of all other living things. They proposed that these microorganisms, which they called archaeobacteria, constitute a primary line of evolutionary descent as distinct from the lineage of other procaryotes (the eubacteria) as it is from the eucaryotic nuclear lineage. Interest in the archaeobacteria has continuously increased as this hypothesis has become more and more widely accepted. In particular, it has been argued that the archaeobacteria afford us a third perspective through which we may be able to identify the characteristics of the common ancestors (progenotes) from which the eubacterial, archaeobacterial, and eucaryotic lineages descended (2, 14).

In recent years it has been increasingly recognized that the study of the archaeobacteria is at an impasse due to the lack of broadly applicable genetic methodologies. Among the archaeobacterial genera, the halobacteria show the most promise for the development of genetic systems because of the ease with which they can be manipulated in the laboratory. Many of the elements which have formed the basis of genetic methodologies in other microorganisms have already been reported for the halobacteria: phages, plasmids, and transposable elements have all been described, and in a number of cases their DNAs have been restriction mapped or sequenced (1, 4). Recently, Mevarech and Werczberger (10) described a system of natural genetic transfer between intact cells of *Halobacterium volcanii*. A method for inducing the uptake and expression of exogenous DNA is also essential, if modern recombinant DNA and in vitro mutagenesis techniques are to be successfully applied to the halophilic archaeobacteria.

The search for archaeobacterial transformation systems has been frustrated by the lack of selectable genetic loci such as might ordinarily be used to score successful transformation events. We have therefore relied on a plaque assay to score for successful transfection of phage DNA in determining conditions for the efficient uptake of DNA by *Halobacterium halobium*. To our knowledge, this is the first demonstration of transfection (or of any sort of uptake and expression of exogenous DNA) in an archaeobacterium.

Media and growth conditions. *H. halobium* R₁ was obtained from W. Zillig. Our usual growth medium contained, per liter, 250 g of NaCl, 20 g of MgSO₄ · 7H₂O, 3 g of trisodium citrate · 2H₂O, 2 g of KCl, 3 g of Bacto Yeast Extract (Difco Laboratories, Detroit, Mich.), and 5 g of Bacto Tryptone (Difco). Plates were made with medium diluted by 20% and containing 15 g of Bacto-Agar (Difco) per liter. Top agar contained 8 g of agar per liter. Spheroplast regeneration plates and spheroplast regeneration top agar contained 15% sucrose as an osmotic stabilizer as recommended by Jarrell and Sprott (3). Solutions of medium salts, yeast extract-Tryptone, agar, or sucrose were autoclaved separately. Liquid cultures were incubated at 37°C on a Junior Orbit Shaker (Lab-Line Instruments, Inc., Melrose Park, Ill.) at 150 rpm. Plate cultures were incubated at 40°C. Transfection efficiency and spheroplast regeneration were comparable when 0.2 g of CaCl₂ per liter was included in media.

Preparation of Φ H DNA. Halophage Φ H, which infects *H. halobium*, was obtained from W. Zillig. Φ H DNA is a linear 59-kilobase-pair molecule with about 2-kilobase-pairs of terminal redundancy (11). Naked DNA was prepared by a modification of a protocol for the rapid isolation of lambda phage DNA (8). Briefly, phage was precipitated from a lysate supernatant with 10% polyethylene glycol (PEG, 8,000 molecular weight) and dissolved in 1 M NaCl–0.1 M MgSO₄. The phage solution was treated with DNase I, reprecipitated with PEG, redissolved in 1 M NaCl–0.1 M MgSO₄, and diluted fivefold with 50 mM Tris hydrochloride (pH 8)–50 mM EDTA–0.1% sodium dodecyl sulfate. Insoluble material was pelleted, and then the supernatant containing the phage DNA was extracted with phenol and chloroform. Phage DNA was precipitated with isopropanol, washed with ethanol, and then dried and suspended in 10 mM Tris hydrochloride (pH 8)–1 mM EDTA. The resulting Φ H DNA migrated as a single discrete band of expected mobility on a 0.35% agarose gel. Concentration was determined by comparison of scanning densitometer traces of the Φ H DNA and λ DNA standards on a photographic negative of the ethidium bromide-stained gel.

Spheroplast formation. Spheroplasts were formed by a modification of the method of Sumper and Herrmann (12). A mid- to late-log-phase culture of *H. halobium* was diluted 50-fold into medium and grown to an optical density (A_{550}) of 1 to 1.5. This corresponds to about 2×10^9 to 3×10^9 cells per ml as determined by viable cell titer. Cells were pelleted

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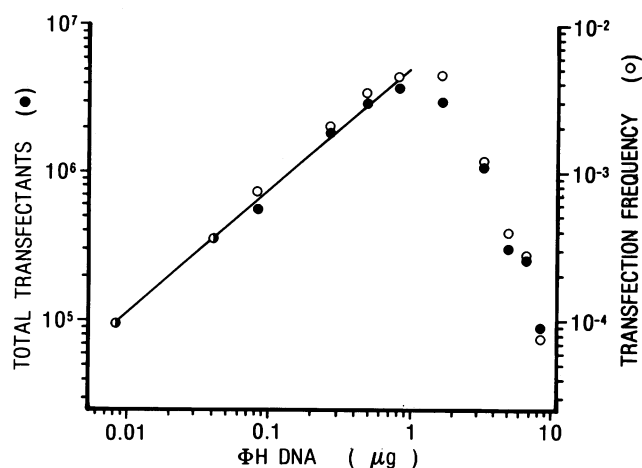


FIG. 1. Dependence of total transfectants (●) or transfection frequency (○) on input Φ H DNA. Transfection frequency is expressed as transfectants per regnerant colony.

in a warm rotor (model GLC-1 centrifuge [Ivan Sorvall, Inc., Norwalk, Conn.] with type SPX rotor) at $3,300 \times g$ for 10 min and then were gently suspended in 1/10 volume of spheroplasting solution (2 M NaCl, 27 mM KCl, 50 mM Tris hydrochloride [pH 8.75], 15% sucrose). A 10- μ l amount of 0.5 M EDTA (in spheroplasting solution) was added to 0.2-ml samples of the concentrated cells with brief, gentle agitation. Cells were completely converted to spheroplasts during 5 to 10 min of incubation at room temperature.

Transfection. After spheroplasts were formed, Φ H DNA was added, typically in a volume of 10 μ l of spheroplasting solution, and the mixture was incubated for 5 min at room temperature. An equal volume of a solution containing 60% PEG 600-40% unbuffered spheroplasting solution (wt/vol) was then added with gentle mixing, and the preparation was incubated for 30 min at room temperature. PEG 600 (polyethylene glycol of approximately 600 molecular weight) was obtained from Sigma Chemical Co., St. Louis, Mo., and purified by the method of Klebe et al. (5).

To plate for scoring transfectants, spheroplasts were gently diluted to 10 ml with spheroplast dilution solution (medium salts plus 15% sucrose). Further dilutions were made as required. Samples (100 μ l) were added to 3 ml of regeneration top agar (maintained at 60°C), along with 200 μ l of late-log-phase plating culture, and then layered on regeneration plates. Φ H plaques arising from transfectants spheroplasts were scored after 5 to 7 days.

We performed several control tests to ensure that plaque formation truly indicated transfection. When Φ H DNA was omitted from the protocol, no plaques were formed; therefore, the transfection protocol did not induce a preexisting phage lysogen. Φ H DNA was completely noninfectious in ordinary phage-plating procedures. When 34 ng of Φ H DNA was pretreated for 30 min at room temperature with 1 μ g of DNase I (Sigma) in 10 μ l of spheroplasting solution (adjusted to pH 7.4 and 5 mM MgSO_4), transfecting activity was completely eliminated. When spheroplasts were treated with Φ H DNA and PEG was omitted, no transfection occurred. These results eliminated the possibility that ordinary phage infection produced the plaques, established the input naked Φ H DNA as the agent of plaque formation, and demonstrated the requirement for PEG in the transfection.

Spheroplast viability. Spheroplast viability was determined

by titring on spheroplast regeneration plates. *H. halobium* spheroplasts, as has been shown for spheroplasts of *Halobacterium cutirubrum* and *Halobacterium salinarum* (3, 9), are capable of growth and eventually regenerate their normal rod-shaped appearance when Mg^{2+} is returned to normal levels. This implies regeneration of the glycoprotein cell envelope (9). Regenerant colonies were scored 10 to 14 days after plating.

Optimization of transfection. The number of transfectants was directly proportional to the amount of input Φ H DNA (from 10 ng to 0.8 μ g) and was equivalent to 5×10^6 transfectants per μ g of Φ H DNA (Fig. 1). At higher DNA concentrations the number of transfectants declined. This appeared to coincide with PEG precipitation of DNA at higher concentrations: precipitation of DNA upon the addition of PEG to transfections was visibly evident at 3 μ g of Φ H DNA and above. This is not surprising given the combined high NaCl and PEG concentrations (7).

Early- or mid-log-phase cultures transfected poorly. Best results were obtained with late-log-phase cells within about 1 doubling of stationary phase, but the number of transfectants declined as the culture approached saturation.

The maximum number of transfectants was obtained at a NaCl concentration around 2 M. Increasing NaCl to 3 M resulted in a 500-fold decrease in transfection frequency. Reducing NaCl to 1 M resulted in a sixfold decrease in spheroplast viability, as well as a decrease in transfection frequency. An increase in the frequency of transfection coincided with a decrease in spheroplast viability between pHs 8.1 and 9.4, giving a maximum number of transfectants between these pHs.

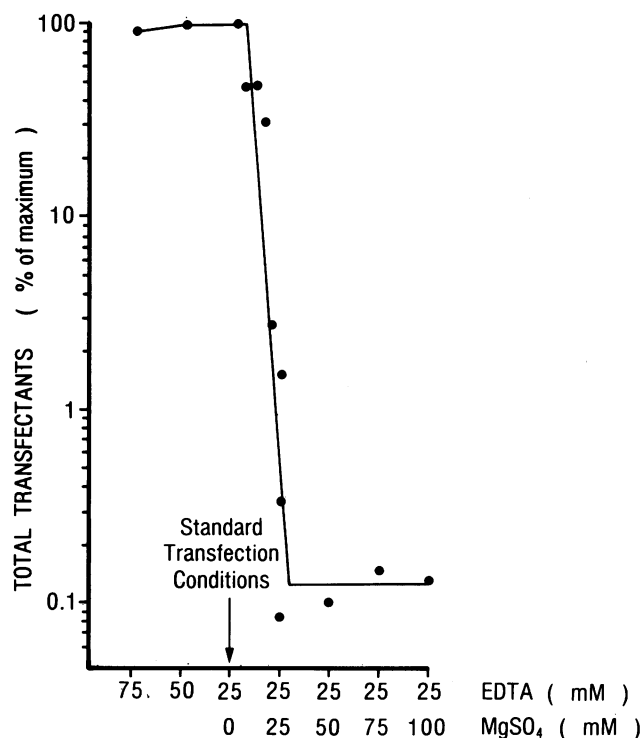


FIG. 2. Effect of EDTA or MgSO_4 concentrations on transfection. Transfectants are expressed as percentages relative to standard transfection conditions (5.8×10^5 transfectants with 25 mM EDTA). All spheroplast mixtures initially contained 25 mM EDTA, and then additional EDTA or MgSO_4 was added. Spheroplasts were transfected with 34 ng of Φ H DNA.

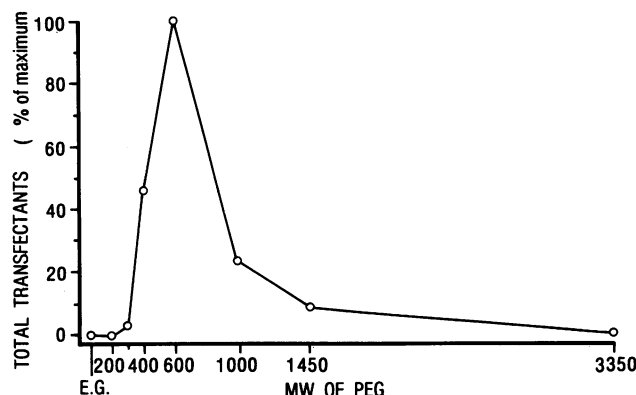


FIG. 3. Effect of molecular weight of PEG on transfection. Transfectants are expressed as percentages relative to standard transfection conditions (3.4×10^5 transfectants with PEG 600). All PEGs were commercially available approximate molecular weight fractions (Sigma) purified by the method of Klebe et al. (5). Ethylene glycol (E.G.), PEG 200, and PEG 8000 (not shown) gave no transfectants ($<0.003\%$). Spheroplasts were transfected with 34 ng of Φ H DNA.

PEG-mediated transfection of *H. halobium* is unusual in that divalent cations reduce transfection efficiency; divalent cations are stimulatory, if not required, for PEG-mediated uptake of DNA in other organisms. For *H. halobium*, initial chelation of Mg^{2+} is required for spheroplast formation, and this is accomplished in our standard protocol by making the cell suspension 25 mM with respect to EDTA. Chelation of divalent cations is also required for efficient transfection: when $MgSO_4$ (in spheroplasting solution) was added back after spheroplast formation, transfection efficiency was reduced 1,000-fold (Fig. 2). $CaCl_2$ had a similar effect (not shown). Divalent cations stabilize halobacterial membranes (6), which suggests one explanation for the reduction in transfection efficiency, but we have not ruled out other

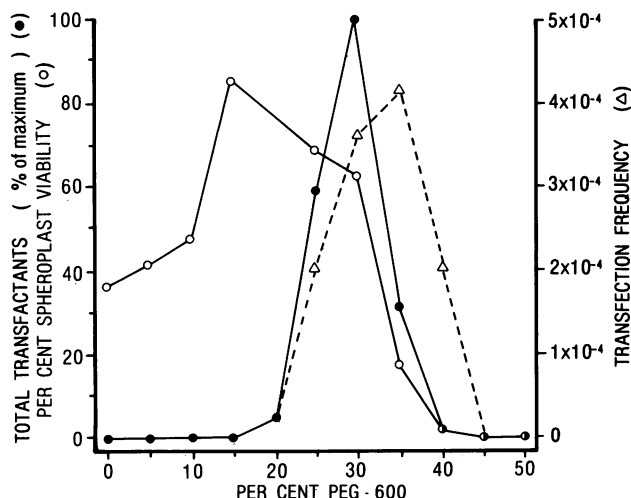


FIG. 4. Effect of percent (wt/vol) PEG 600 in transfection mixture. Transfectants (\bullet) are expressed as percentages relative to standard transfection conditions (8.7×10^5 transfectants at 30% PEG 600). Spheroplast viability (\circ) is expressed as the percentage of regenerant colonies relative to the original culture titer. Transfection frequency (Δ) is expressed as transfectants per regenerant colony. Spheroplasts were transfected with 34 ng of Φ H DNA.

possibilities such as divalent cation-dependent nuclease activity. Additional EDTA up to 75 mM did not significantly affect transfection or spheroplast viability; 20 mM or greater $MgSO_4$ improved spheroplast viability slightly.

When we varied PEG molecular weight in our standard protocol, PEG-600 was found to be the most effective (Fig. 3). Spheroplast viability was not significantly affected by the molecular weight of the PEG. We are unaware of any other PEG-mediated transfection-transformation protocol in which PEG of such low molecular weight is optimal. We observed a sharp maximum near 30% for the most effective final PEG concentration in the transfection mixture (Fig. 4). This maximum nearly coincided with an optimum for transfection frequency at 35% PEG. We also varied the length of incubation with PEG and found that a 30-min PEG treatment gave 15% more transfectants than did a 10-min treatment and 75% more than did a 5-min treatment. The number of transfectants remained essentially unchanged between 30 min and 1 h of PEG treatment.

Regeneration efficiency and transfection frequency. In the experiment summarized in Fig. 1, about 20% of the input cells survived the transfection procedure and regenerated to form normal colonies. The percentages of surviving regenerants varied somewhat between batches of cells: 60 to 75% was not unusual. When the survival rate was higher, the number of transfectants was concomitantly higher. For example, in the experiment shown in Fig. 4 (30% PEG), the transfection procedure gave 62% spheroplast survival and 8.7×10^5 plaques when 34 ng of Φ H DNA was added. This extrapolates to 2.5×10^7 transfectants per μ g of DNA. The right-hand scale of Fig. 1 indicates the transfection frequency as transfectants per regenerant colony. Transfectants were about 0.5% of the surviving spheroplasts at a level of 1 μ g of Φ H DNA. In the experiment shown in Fig. 4 (30% PEG), the transfection frequency extrapolated to 1.1% of surviving spheroplasts at the 1- μ g level. These regeneration efficiencies and transfection frequencies were sufficiently high to suggest that this method of inducing the uptake and expression of exogenous DNA in *H. halobium* may be applicable to other DNAs such as plasmids. The transfection frequencies are easily in a range in which even nonselective screening for transformants (e.g., by colony hybridization) should not be difficult.

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ADDENDUM IN PROOF

Using an indicator lawn of *H. halobium* R₁, we have recently shown that *H. volcanii*, which is outside the host range of halophage Φ H, can also be transfected by Φ H DNA using our method.

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